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Endocannabinoid Signaling Directs Periimplantation Events

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ABSTRACT

An emerging concept in female reproduction is the role of endocannabinoids, a group of endogenously produced lipid mediators that bind to and activate cannabinoid receptors. Although adverse effects of cannabinoids in female reproduction have been implicated for years, the mechanisms by which they exert these effects remained elusive. With the identification of cannabinoid receptors, endocannabinoid ligands, their key synthetic and hydrolytic pathways, and the generation of knockout mouse models for cannabinoid receptors, a wealth of information is now available regarding the significance of cannabinoid/endocannabinoid signaling in early pregnancy. This review focuses on various aspects of endocannabinoid signaling in preimplantation embryo development and activation, and uterine differentiation during the periimplantation embryo-uterine dialog. It is hoped that a deeper understanding will lead to potential clinical applications of the endocannabinoid system as a target for regulating female fertility.

KEYWORDS: cannabinoid/endocannabinoid, embryo development, embryo oviductal transport, implantation, mouse

INTRODUCTION

Synchronous development of the preimplantation embryo to the blastocyst stage and differentiation of the uterus to the receptive stage are prerequisites for the initiation of implantation in all species.[1-3] A considerable number of early pregnancy losses occur because of either preimplantation embryonic death or implantation failure resulting from asynchronous embryonic development and/or failure of the uterus to differentiate to the receptive stage.[4,5] Understanding the mechanism of preimplantation embryonic development and implantation in the uterus is a challenge to reproductive biologists with the goal of alleviating the problems of human infertility and ensuring the birth of quality offspring. Such knowledge would also help in developing novel contraceptive approaches to restrict rapidly growing world population. Although details of many of the molecular interactions during the periimplantation pregnancy event have not yet been defined, increasing evidence from gene expression and transgenic mouse studies reveals that coordinated integration of a range of signaling pathways via paracrine, autocrine, and/or juxtacrine manners participates in embryo-uterine dialog during implantation.[1-3,6,7] Among these signaling pathways, the endocannabinoid signaling has recently been highlighted as an important player in directing preimplantation embryo development and their timely homing into the receptive uterus for implantation.

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PREIMPLANTATION EMBRYO IS A TARGET FOR CANNABINOID/ENDOCANNABINOID SIGNALING

Δ^9-Tetrahydrocannabinol (THC), the major psychoactive component in marijuana, exerts a wide array of adverse effects on human health, including reproduction. With the identification of 2 types of cannabinoid receptors, brain-type CB1 and spleen-type CB2,[8,9] and 2 major endocannabinoids, *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG)[10-12] as ligands for these receptors in the early 1990s, our laboratory (using mice as an animal model) has been pursuing studies on the pathophysiological significance of endocannabinoid ligand-receptor signaling during early pregnancy.

Cannabinoid Receptors in the Preimplantation Embryo and Uterus

In mice, only CB1 is expressed in the oviduct and uterus, while both CB1 and CB2 are expressed in preimplantation embryos.[13-16] CB1 mRNA is detected from the 4-cell through the blastocyst stages, whereas that of CB2 is present from the 1-cell through the blastocyst stages.[14] AEA binding sites are also evident at these stages. Of interest,

these binding sites are mostly present in outer cells of embryos at 8-cell, morula, and blastocyst stages. Scatchard analysis of binding kinetics in blastocysts showed that AEA binds to a single class of high affinity receptors. The presence of CB1 mRNA correlates with CB1 protein as detected by immunocytochemistry.[15,17] Furthermore, blastocyst CB1 is biologically active, since both THC and anandamide inhibit forskolin-stimulated cAMP formation, and this inhibition is prevented by pertussis toxin pretreatment.[13,14] The presence of functional CB1 in the blastocyst suggests that the mouse embryo is a target for endocannabinoids and natural cannabinoids. In fact, 2-cell embryos fail to develop into blastocysts when exposed to synthetic (CP 55940, WIN 55212-2), natural (THC), or endocannabinoids (AEA and 2-AG) in culture.[14,18] A reduction in trophectoderm cell numbers is noted in those blastocysts that escaped the developmental arrest in the presence of cannabinoid agonists.[17] However, these adverse effects are reversed by simultaneous addition of a selective antagonist to CB1 (SR 141716[19]) with cannabinoid agonists, but not by a selective CB2 antagonist (SR 144528[20]).[18] Recent observations of CB2 expression in early embryos and embryonic stem cells by microarray analysis,[21] and the absence of its expression in trophoblast stem cells derived from preimplantation blastocysts (H. Wang and S.K. Dey, unpublished data, 2006) suggest that CB2 expression is restricted to blastocyst inner cell mass (ICM) cells. These studies suggest that although the role of CB2 in the early embryo is not known, cannabinoids/endocannabinoids can influence preimplantation embryo development via CB1.

Asynchronous Embryo Development in Cannabinoid Receptor Mutant Mice

We surmised that if a tight regulation of anandamide ligand-receptor signaling is important for early embryo development, embryos deficient in cannabinoid receptors would exhibit asynchronous development. With the availability of cannabinoid receptor knockout mice,[22,23] the physiological relevance of cannabinoid receptor signaling during early embryo development was further examined. We observed that *CB1−/−*, *CB2−/−*, or *CB1−/−*x*CB2−/−* double-mutant embryos recovered from the oviduct on day 3 and from the uterus on day 4 of pregnancy show asynchronous development compared with wild-type embryos.[15,16] This aberrant embryo development is rescued by mating *CB* mutant females with wild-type males that produce all heterozygous embryos in a mutant maternal environment. This finding indicates that embryonic CB receptors, but not maternal factors, direct early synchronous embryonic development.[16] These studies also provide evidence that CB1 and CB2 are critical for preimplantation embryo development, although asynchronous development of *CB2−/−* embryos still remains puzzling. Since CB2 is expressed in the embryonic stem cells, but not in trophectoderm-derived trophoblast stem cells, it is conceivable that CB2 plays a role in specifying pluripotent ICM cell lineage during blastocyst formation. To determine whether embryos deficient in CB receptors respond to endocannabinoids in vitro, 2-cell wild-type or mutant embryos were cultured in the presence or absence of AEA. While a comparable development of wild-type and mutant embryos was observed in the absence of AEA, *CB1−/−* and *CB1−/−*x*CB2−/−* mutant embryos, but not *CB2−/−* or wild-type embryos, were resistant to anandamide's inhibitory action.[15] This observation reinforces CB1 as the functional receptor for ensuring normal embryo growth and differentiation to blastocysts. Collectively, these studies provide pharmacological, molecular, and genetic evidence that the preimplantation embryo is indeed a target for cannabinoid/endocannabinoid signaling.

Endocannabinoid Signaling Guides Embryo Transport

During early pregnancy, another critical event occurring in parallel with preimplantation embryonic development is the embryos' timely transport from the oviduct into the uterus. In mice, embryos at the late morula or early blastocyst stage enter the uterus, where they develop and differentiate to gain implantation competency, escape from the zona pellucida, and implant into the receptive uterus. Thus, normal oviductal embryo transport is one of the prerequisites for on-time implantation, whereas a dysfunctional regulation of this process resulting from oviductal embryo retention may increase the incidence of pregnancy failure or cause tubal pregnancy in humans.

Aberrant Cannabinoid Signaling, Silencing, or Amplification Derails Oviductal Embryo Transport

During the course of our study over the past several years, exploring the potential physiological roles of endocannabinoid signaling during early pregnancy, we consistently observed that ~40% of *CB1−/−* mice show pregnancy loss.[15,16] Since these mutant mice have normal ovulation and fertilization,[16] we initially thought that asynchronous embryo development could be the major cause of this pregnancy loss. We speculated that normal pregnancy in *CB1−/−* mice would be restored by mating mutant females with wild-type males to generate all heterozygous embryos with normal preimplantation growth. However, we still observed that ~40% of *CB1−/−* mothers failed to yield any embryos in the uterus when examined on day 4 midmorning, suggesting that a maternal CB1, but not embryonic CB1, is the cause for pregnancy failure.

To determine the underlying cause for this pregnancy failure, we examined oviductal embryo transport in *CB1−/−*,

CB2−/−, and *CB1−/−*x*CB2−/−* double-mutant females. No embryos were found to be trapped in oviducts of wild-type or *CB2−/−* mice, which was expected since only CB1 is expressed in the mouse oviduct and uterus. However, a substantial number of *CB1−/−* and *CB1−/−*x*CB2−/−* mice showed impaired oviductal transport with retention of embryos at the morula and blastocyst stages within the oviduct on day 4 (Figure 1). These trapped embryos appeared morphologically normal and implanted upon transfer into day 4 pseudopregnant recipient uteri, suggesting that they remained implantation competent. These results indicate that maternal expression of CB1 in the reproductive tract plays an important role in normal oviduct to uterine transport of embryos, and its deficiency results in embryo retention in the oviduct for an extended period, causing reduced fertility in *CB1−/−* mice. This observation was confirmed in our reciprocal embryo transfer experiments between *CB* mutant and wild-type mice. Indeed, only *CB1−/−* recipients showed oviductal embryo retention and implantation failure, irrespective of the genotypes of donor embryos.[16] We further observed that wild-type pregnant mice treated with a CB1 selective antagonist, SR141716, exhibited impaired embryo transit through the oviduct, but this defect did not occur in mice treated with vehicle or a CB2 selective antagonist, SR144528. Of interest, wild-type females exposed to a stable anandamide analog (methanandamide) or natural THC, showed pregnancy loss with embryos retained in the oviduct.[16] These observations demonstrate that aberrant cannabinoid signaling, either silenced or enhanced, impairs embryo transport. This suggests that there is an endocannabinoid tone mediated via CB1 in the oviduct that regulates normal embryo transport into the uterus for implantation. Collectively, these observations show that while embryonic CB1 primarily contributes to normal embryo development, oviductal CB1 directs timely oviductal transport of embryos.

Coordinated Cannabinoid-adrenergic Signaling Ensures Normal Embryo Journey From Oviducts Into Uteri

Previous studies have established that the embryo's journey from the oviductal isthmus into the uterus in rodents is regulated by a wave of coordinated contraction and relaxation of the oviduct muscularis. It is believed that the sympathetic neuronal circuitry, under the direction of ovarian hormones, coordinates the "closing and opening" of the sphincter at the isthmus-uterine junction, thereby regulating the timely transit of embryos from the oviduct into the uterus.[24,25] During pregnancy, rising progesterone levels from the newly formed corpora lutea decrease the levels of noradrenaline, which shows higher affinity for α-adrenergic receptors (AR) than the β-AR at the adrenergic nerve endings.[26] In contrast, the sensitivity of the β-AR is increased in the circular muscle of the oviduct isthmus under progesterone dominance, causing muscle relaxation and facilitating embryo transport through the oviduct.[24] The observation of embryo retention within the oviduct in wild-type females after exposure to an α1-AR agonist phenylephrine and/or a β2-AR antagonist butoxamine[16] led us to speculate that CB1-mediated endocannabinoid signaling is functionally coupled to adrenergic signaling to regulate oviductal motility conducive to embryo transport. This speculation is consistent with colocalization of CB1 with that of α1-AR and β2-AR in mouse oviductal muscularis at the isthmus region, and the restoration of normal embryo transport in *CB1−/−* mice by β-AR agonist isoproterenol.[16] Experiments with in vitro [^{3}H]noradrenaline release further provided evidence that genetic or pharmacological

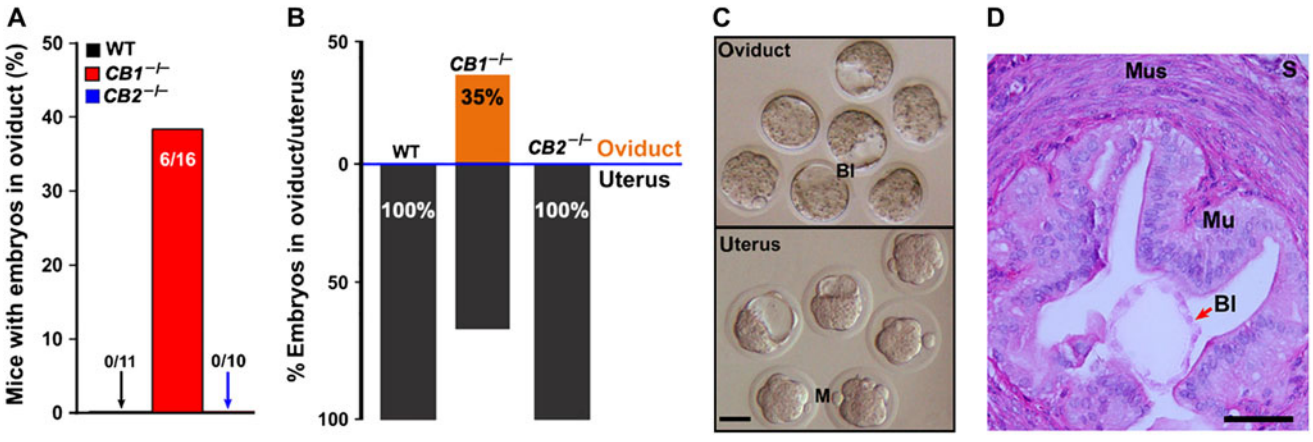

Figure 1. Impaired oviductal embryo transport causes pregnancy loss in *CB1−/−* mice: (A) number of mice with oviductal retention of embryos/total number of mice examined; (B) percentage of embryos recovered from oviducts or uteri; (C) morphology of embryos recovered from oviducts or uteri. Both morulae and blastocysts are recovered from oviducts or uteri of *CB1−/−* mice on day 4 midmornings (bar, 50 μm); (D) a representative histological section of a day 7 pregnant *CB1−/−* oviduct showing a trapped blastocyst (Bl, arrow) at the isthmus (bar, 100 μm). WT indicates wild-type; Bl, blastocyst; Mus, muscularis; S, serosa; Mu, mucosa; CB1, brain-type cannabinoid receptor. Reprinted with permission from *Nature Medicine*.[16]

loss of functional oviductal CB1 increases noradrenaline release from the adrenergic nerve terminals, maintaining a smooth muscle contractile tone through α-AR, and thereby impeding oviductal embryo transport. In contrast, exposure to excessive natural or synthetic cannabinoid ligands leads to predominant relaxation phase of the oviductal muscularis owing to attenuated noradrenaline release, impairing embryo transport.[16] Collectively, these findings reinforce the concept that an aberrant cannabinoid signaling, either silenced or amplified, impedes the highly coordinated oviductal smooth muscle contraction and relaxation critical to embryo transport during early pregnancy. The potential mechanism creating this endocannabinoid tone in the oviduct during early pregnancy remains to be explored.

Biphasic Endocannabinoid Signaling Coordinates Blastocyst Activation and Uterine Receptivity for Implantation

The preimplantation events of early embryo development and transport are synchronized with the proliferation and differentiation of specific cells of the uterus, primarily under the direction of ovarian estrogen and progesterone. These hormones make the uterus conducive (receptive) to accept an implantation-competent (activated) blastocyst for implantation.

Ovarian Progesterone and/or Estrogen Sets up Blastocyst-Uterine Dialog for Implantation

Progesterone has shown to be essential for implantation and pregnancy maintenance in all mammals studied, whereas requirement for ovarian estrogen is species specific. For example, ovarian progesterone and estrogen are essential to implantation in mice and rats, but ovarian estrogen is dispensable in pigs, guinea pigs, rabbits, and hamsters. However, estrogen produced by embryos is considered important for implantation in the latter species.[1]

In mice, under progesterone priming, the closure of the uterine lumen occurs and coincides with the escape of the blastocyst from the zona pellucida, bringing the blastocyst trophectoderm in close contact with the uterine luminal epithelium (apposition). Superimposition of the progesterone-primed uterus with preimplantation ovarian estrogen secretion and its catechol metabolite, 4-hydroxy-17β-estradiol (4-OH-E_2), produced from primary estrogen in the uterus differentially regulate uterine preparation and blastocyst activation, respectively. For example, the primary estrogen, via its interaction with nuclear estrogen receptors, participates in the preparation of the progesterone-primed uterus to the receptive state in an endocrine manner, whereas its metabolite, 4-OH-E_2, mediates blastocyst activation for implantation in a paracrine manner.[27] These coordinated actions of progesterone and estrogen set up the window of implantation. One of the major events in the process of implantation is the attachment of the blastocyst trophectoderm with the uterine luminal epithelium that occurs within a narrow window of time frame resulting from an intimate 2-way dialog between the implantation-competent blastocyst and the receptive uterus. In mice, this attachment reaction is initiated around midnight on day 4 of pregnancy.[28] However, elimination of preimplantation estrogen secretion by ovariectomy on the morning of day 4 results in implantation failure and blastocyst dormancy within the quiescent uterine lumen.[29,30] This condition is referred to as delayed implantation and can be maintained for many days by continued progesterone treatment. However, implantation with blastocyst activation is rapidly initiated by a single injection of estrogen in the progesterone-primed uterus.[29,30] This physiologically relevant delayed implantation model is widely being used to identify signaling pathways mediating embryo-uterine cross-talk during implantation. We have recently elucidated a unique association of endocannabinoid signaling with embryo-uterine interactions during implantation.

Blastocyst CB1 and Uterine AEA Levels Are Downregulated With the Onset of Activation Before implantation

As described earlier, natural, synthetic, or endocannabinoids inhibit preimplantation embryo development and blastocyst zona-hatching in culture.[14,18,31] This observation correlates well with higher levels of AEA in the nonreceptive uterus.[15,31,32] On the other hand, lower levels of AEA in the receptive uterus and at the implantation site suggest that regulated AEA levels are conducive to normal embryo development and implantation. There is evidence that cannabinoid effects are differentially executed depending on the embryonic stage and cannabinoid levels. Blastocysts exposed in culture to low levels of AEA exhibit accelerated trophoblast differentiation and outgrowth, while inhibition of trophoblast differentiation is observed at higher doses of AEA,[33,34] suggesting dual functions of AEA depending on its local concentration.[31,33] Thus, uterine AEA levels are critical in regulating the "window" of implantation by synchronizing trophoblast differentiation and uterine preparation to the receptive state.

To gain further insight into the underlying causes of these biphasic effects of AEA, the status of anandamide binding in pre-attachment and attachment-competent blastocysts immediately prior to implantation on day 4 of pregnancy was studied. Similar studies were also performed using dormant and estrogen-activated blastocysts. The results showed that normal blastocysts collected on the early morning of day 4 had higher levels of AEA binding, but this binding

remarkably declined in blastocysts recovered on day 4 in the late afternoon prior to the attachment reaction.[15] These observations suggested that downregulation of AEA binding to the blastocyst is important for achieving implantation competence. Similarly, dormant blastocysts also showed increased levels of AEA binding sites, but this binding significantly decreased by 12 hours after termination of dormancy by an estrogen injection.[15] The immunoreactive CB1 protein paralleled AEA binding in dormant and activated blastocysts (Figure 2).[15,35] These results collectively suggest that coordinated downregulation of blastocyst CB1 and uterine AEA levels in the receptive uterus are important for implantation.

Maternal N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) and Embryonic Fatty Acid Amide Hydrolase (FAAH) Are Primary Determinants of Uterine Anandamide Levels During Implantation

Increasing evidence suggests that the bioeffectiveness of AEA depends on its concentration in the extracellular space, which is regulated by its intracellular synthesis by NAPE-PLD and degradation by fatty acid amide hydrolase (FAAH).[36-38] To further address the underlying mechanism by which differential uterine AEA levels are spatiotemporally established under different pregnancy status, we examined the expression profiles of NAPE-PLD and FAAH in the mouse uterus during early pregnancy. Correlating with higher levels of AEA in the nonreceptive uterus and interimplantation sites, higher levels of *Nape-pld* mRNA and NAPE-PLD activity were detected at these sites as compared with implantation sites and receptive uteri (Figure 3).[32] It is interesting to note that the implanting blastocyst exerts an inhibitory effect on uterine *Nape-pld* expression.[32] There is also evidence that blastocysts can upregulate uterine FAAH activity by releasing a lipid "FAAH activator."[39] These observations suggest a potential role of the implanting embryo in regulating uterine AEA levels, perhaps to serve as a protective mechanism against exposure to detrimental levels of AEA. This is further confirmed by the observation of higher FAAH expression and activity in the implanting embryo.[40,41] Therefore, differential and dynamic expression and activity of NAPE-PLD and FAAH in the embryo and uterus create optimal levels of AEA beneficial to blastocyst activation and uterine receptivity for implantation. This tight regulation of AEA synthesis and hydrolysis in the pregnant uterus further indicates that endocannabinoid signaling plays an important role in implantation. These studies clearly establish the concept that while lower levels of AEA and CB1 are beneficial for implantation, higher levels are detrimental to this process.

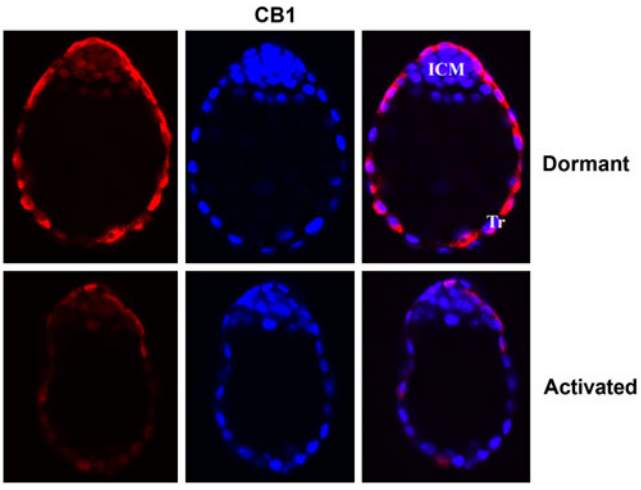

Figure 2. Dramatically upregulated CB1 protein in dormant blastocyst Tr cells and its rapid downregulation with the termination of delayed implantation with blastocyst activation by estrogen. CB1 is upregulated in dormant blastocysts. The trophectoderm (Tr) cell surface is decorated with CB1. Images shown depict antigens in red and Hoechst-labeled nuclei in blue. ICM indicates inner cell mass; CB1, brain-type cannabinoid receptor. Reprinted with permission from *Proceedings of the National Academy of Sciences of the United States of America*.[35]

Differential CB1 Signaling by Anandamide Directs Blastocyst Activation for Implantation

Using the delayed implantation mouse model, we have provided further evidence that AEA at low concentrations confers blastocyst competency to implantation via CB1,[35] whereas experimentally elevated natural or synthetic cannabinoid levels interfere with normal pregnancy. These findings are consistent with our previous observations of stimulation and inhibition of trophoblast growth at low and high AEA levels, respectively.[33] To reveal the underlying mechanism of this biphasic AEA action in blastocyst implantation, we further explored the potential signaling pathways that are coupled with CB1 under different AEA concentrations. We found that AEA-induced stimulatory and inhibitory influences on blastocyst function and implantation are executed by different signal transduction pathways: the extracellular-regulated kinase (ERK) and Ca^{2+} signaling pathways (Figure 4). Anandamide at a low concentration activates ERK signaling in dormant blastocysts via CB1. In contrast, at higher AEA levels, it fails to achieve ERK activation but instead inhibits Ca^{2+} mobilization.[35,42] This finding provides evidence for the first time for a potential "cannabinoid sensor" mechanism to influence crucial steps during early pregnancy. An association of spontaneous pregnancy loss with elevated peripheral AEA levels in women[43,44] is consistent with our observations in mice. These findings in mice and humans reinforce the concept that endocannabinoid signaling is at least one of the pathways determining the fate of embryo implantation.

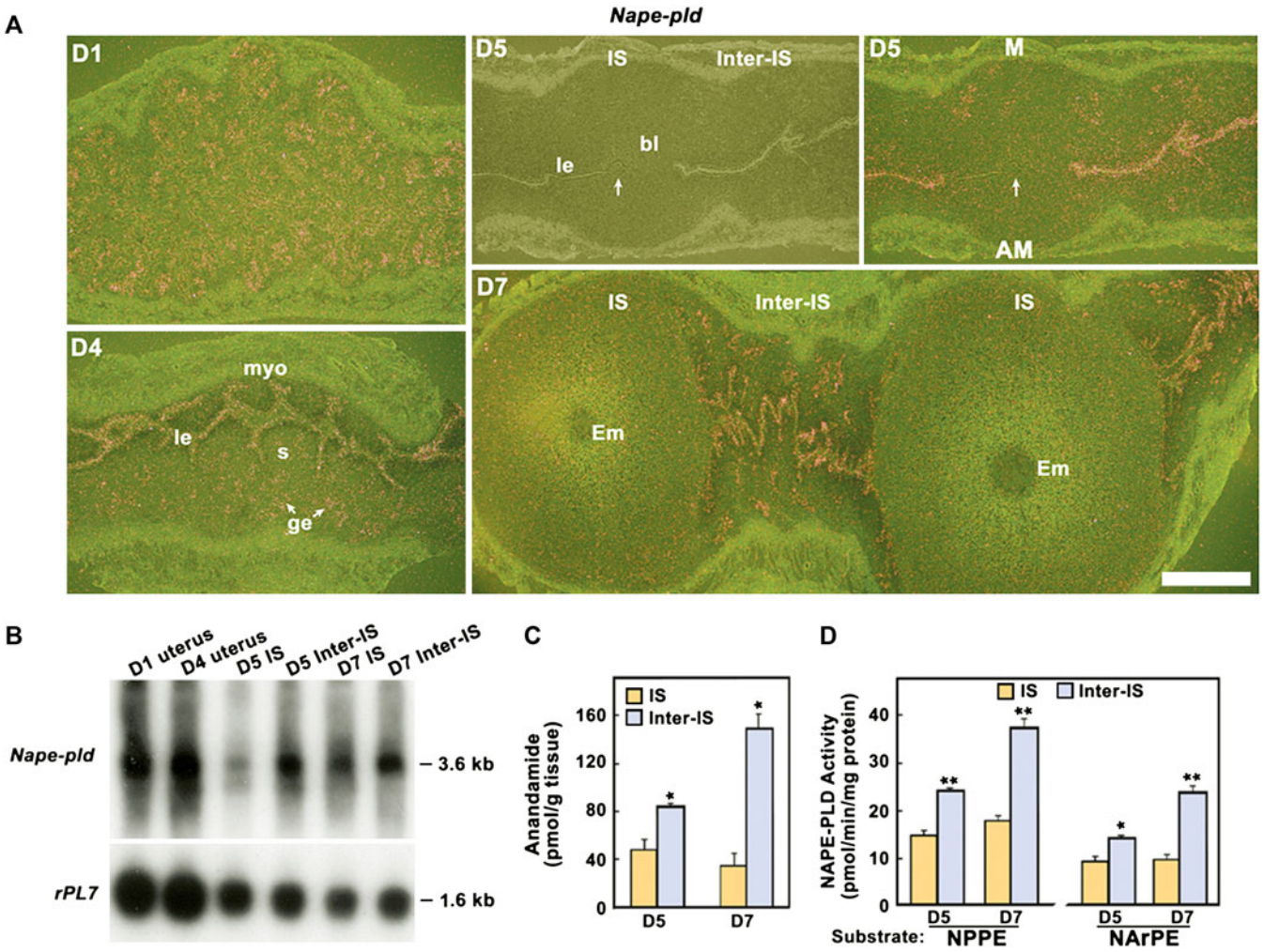

Figure 3. NAPE-PLD determines uterine anandamide levels during implantation. Uterine expression of *Nape-pld* was analyzed by in situ (A) and Northern (B) hybridization. Photomicrographs of representative longitudinal uterine sections are shown in A (bar, 250 µm). C and D, uterine anandamide levels correlate with NAPE-PLD activity during implantation. Higher anandamide levels (C) and NAPE-PLD activity (D) are noted at interimplantation site versus implantation site (unpaired *t* test; *, $P < .05$ and **, $P < .005$). le indicates luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; bl, blastocyst; Em, embryo; IS, implantation site; Inter-IS, interimplantation site; M, mesometrial site; AM, antimesometrial site. Reprinted with permission from *The Journal of Biological Chemistry*.[32]

Conclusion

In this review, we present molecular, genetic, physiological, and pharmacological evidence that cannabinoid/endocannabinoid signaling is functionally operative in early pregnancy events. Our studies demonstrate that under normal physiological conditions, endocannabinoid signaling through CB1 is crucial to various female reproductive events, which include development of embryos, their oviductal transport, and ultimately their homing and implantation in the receptive uterus; conversely, an aberration in endocannabinoid signaling, either silenced or enhanced, derails these processes. Our study adds a new dimension to the concern that the adverse effects on offspring of maternal use of cannabinoids may be seeded very early in pregnancy. There is now evidence that defective implantation creates an adverse ripple effect during the subsequent course of pregnancy in humans and mice.[45-47] Therefore, our findings in mice raise a cautionary note for women of reproductive ages regarding chronic abuse or medicinal consumption of marijuana or other endocannabinoid system-oriented drugs. More important, it raises caution against the use of CB1 antagonists to treat obesity in humans. Further in-depth investigation is warranted to better understand pathophysiological significance of cannabinoid/endocannabinoid signaling pathway in mammalian reproduction.

Acknowledgments

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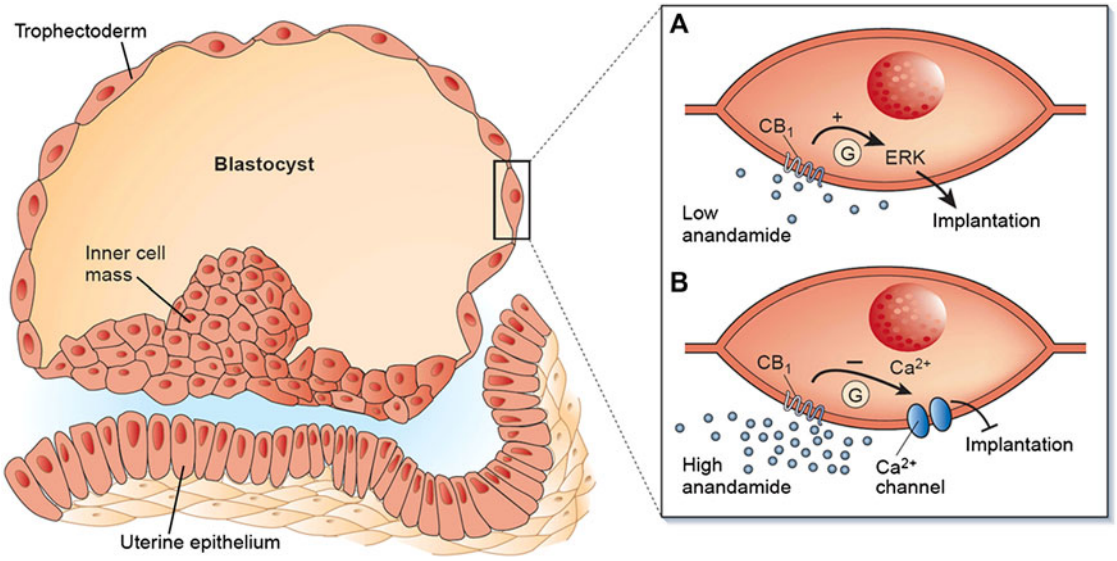

Figure 4. Cannabinoid control of mouse embryo implantation: (A) at low concentrations, the endogenous cannabinoid anandamide may activate cannabinoid receptors (CB1) on the surface of trophectoderm cells, stimulating ERK and facilitating implantation; (B) at higher concentrations, anandamide may engage a second CB1-dependent pathway, which may inhibit the activity of voltage-operated N-type calcium channels, reduce calcium entry, and halt implantation. CB1 indicates brain-type cannabinoid receptor. Reprinted with permission from *Nature Medicine*.[42]

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